

EUROPEAN PATENT APPLICATION

(11) Publication number: A 61 K 31/365  
C 07 D 313/00, C 12 P 17/08  
Int. Cl. 3: A 61 K 31/365  
C 07 D 313/00, C 12 P 17/08  
// (C12P17/08, C12R1/54)

(21) Application number: 81302964.2

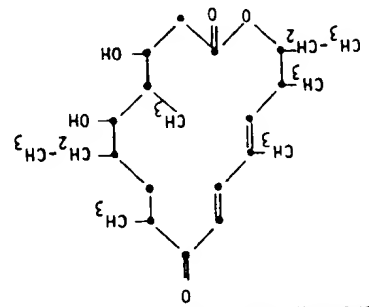
(22) Date of filing: 30.06.81

(30) Priority: 02.07.80 US 162977  
(43) Date of publication of application: 06.01.82 Bulletin 82/1  
(84) Designated Contracting States: AT BE CH DE FR GB IT LU NL SE

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(54) Process for preparing a macrolide.

(57) A process for preparing tyllactone (20-dihydro-20,23-dideoxytyllactone), which has the formula:

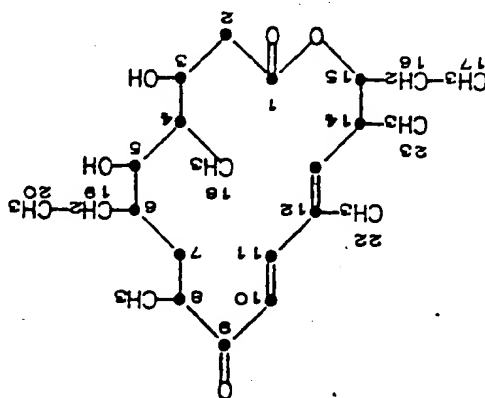


by submerged aerobic fermentation of *Streptomyces fradiae* NRRL 12188 or a tyllactone-producing mutant or recombinant thereof.

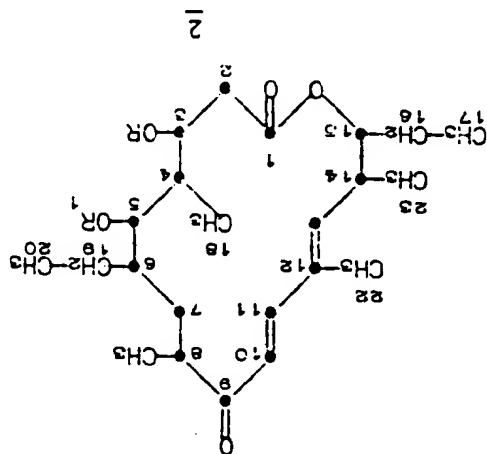
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## PROCESS FOR PREPARING A MACROLIDE

This invention relates to a process for the preparation of the macrolide 20-dihydro-20,23-dideoxy-tylonolide, which will be called tylactone for convenience hereinafter. Tylactone has the structure  $\bar{1}$ :



It is useful in the preparation of related acyl derivatives which have structure  $\bar{2}$ :



wherein R and  $R_1$  = an acyl moiety.

The compounds of structures 1 and 2 are  
 useful intermediates from which 16-membered macroli-  
 de antibiotics can be prepared. Although no stereochem-  
 ical assignments are indicated in the structures given  
 herein, the stereochemistry of the compounds is iden-  
 tical to that of tylosin.  
 Tylosone can be esterified at the 3- and  
 5-hydroxyl groups to give acyl ester derivatives by  
 treatment with acylating agents using methods known in  
 the art. The acyl ester derivatives of tylosone are  
 useful as intermediates in the preparation of new  
 macroli- de antibiotics.  
 Typical acylating agents include anhydrides,  
 halides (usually in combination with a base or other  
 acid scavenger) and active esters of organic acids.  
 Acylation can also be achieved by using a mixture of an  
 organic acid and a dehydrating agent such as N,N'-  
 dicyclohexylcarbodiimide. Acylations can also be  
 carried out enzymatically using procedures such as  
 those described by Okamoto et al. in U.S. 4,092,473.  
 Once formed, the acyl derivatives can be separated and  
 purified by known techniques.  
 The derivatives can be prepared by esterifi-  
 cation techniques generally known in the art, such  
 as, for example, treatment of the compound with a  
 stoichiometric quantity (or a slight excess) of an  
 acylating agent, such as an acyl anhydride, in an  
 organic solvent (for example, pyridine) at about 0°C to  
 about room temperature for from about 1 to about 24  
 hours until esterification is substantially complete.

The ester derivative can be isolated from the reaction mixture by standard procedures such as extraction, chromatography and crystallization. Useful esters are those of organic acids including aliphatic, cycloaliphatic, aryl, aralkyl, heterocyclic carboxylic, sulfonic and alkoxycarbonic acids of from 1 to 18 carbon atoms, and of inorganic acids, such as sulfuric and phosphoric acids. Representative suitable esters include those derived from acids such as formic, acetic, chloroacetic, propionic, butyric, isovaleric, glucuronic, alkoxycarbonic, stearic, cyclopropanecarboxylic, cyclohexanecarboxylic,  $\beta$ -cyclohexylpropionic, 1-adamantanecarboxylic, benzoic, phenylacetic, phenoxyacetic, mandelic and 2-thienylacetic acids, and alkyl-, aryl-, and aralkyl-sulfonic acids, the aryl- and aralkyl-acids optionally bearing substituents such as halogen, nitro, lower alkoxy and the like on the aromatic moiety. Suitable esters also include hemiesters derived from dicarboxylic acids such as succinic, maleic, fumaric, malonic and phthalic acids.

Ty lactone can be prepared by culturing a strain of *Streptomyces fradiae* which produces this compound under submerged aerobic conditions in a suitable culture medium until a substantial amount of the desired compound is produced.

The culture medium used to grow the *Streptomyces fradiae* can be any one of a number of media. For product isolation, however, certain culture media are preferred. Thus, for example, preferred carbon sources

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in large-scale fermentation include carbohydrates such as dextrin, glucose, starch, and corn meal and oils such as soybean oil. Preferred nitrogen sources include corn meal, soybean meal, fish meal, amino acids and the like. Among the nutrient inorganic salts which can be incorporated in the culture media are the customary soluble salts capable of yielding iron, potassium, sodium, magnesium, calcium, ammonium, chloride, carbonate, sulfate, nitrate, and like ions. Essential trace elements necessary for the growth and development of the organism should also be included in the culture medium. Such trace elements commonly occur as impurities in other constituents of the medium in amounts sufficient to meet the growth requirements of the organism. It may be necessary to add small amounts (i.e. 0.2 ml/L) of an antifoam agent such as polypropylene glycol (M.W. about 2000) to large-scale fermentation media if foaming becomes a problem.

For production of substantial quantities of ty lactone submerged aerobic fermentation in tanks is preferred. Small quantities of ty lactone may be obtained by shake-flask culture. Because of the time lag in production commonly associated with inoculation of large tanks with the spore form of the organism, it is preferable to use a vegetative inoculum. The vegetative inoculum is prepared by inoculating a small volume of culture medium with the spore form or mycelial fragments of the organism to obtain a fresh, actively growing culture of the organism. The vegetative inoculum is then transferred to a larger tank. The

medium used for the vegetative inoculum can be the same as that used for larger fermentations, but other media can also be used.

The method of this invention comprises culturing a new microorganism which was obtained by chemical mutagenesis of a Streptomyces fradiae strain which produces tylosin. The new microorganism produces only minimal amounts of tylosin, but produces ty lactone as a major component.

This invention also relates to the new microorganism which produces ty lactone. The new microorganism is also classified as a strain of Streptomyces fradiae. A culture of this microorganism has been deposited and made part of the stock culture collection of the Northern Regional Research Center, Agricultural Research, North Central Region, 1815 North University Street, Peoria, Illinois, 61604, from which it is available to the public under the accession number NRRL 12188.

As is the case with other organisms, the characteristics of Streptomyces fradiae NRRL 12188 are subject to variation. For example, recombinants, mutants or variants of the NRRL 12188 strain may be obtained by treatment with various known physical and chemical mutagens, such as ultraviolet light, X-rays, gamma rays, and N-methyl-N'-nitro-N-nitrosoguanidine. All natural and induced variants, mutants and recombinants of Streptomyces fradiae NRRL 12188 which retain the characteristic of ty lactone production are a part of this invention.

*S. fradiae* NRRL 12188 can be grown at temperatures between about 10° and about 40°C. Optimum production of tylactone appears to occur at temperatures of about 28°C.

As is customary in aerobic submerged culture processes, sterile air is bubbled through the culture medium. For efficient antibiotic production the percent of air saturation for tank production should be about 30% or above (at 28°C and one atmosphere of pressure).

Production of tylactone can be followed during the fermentation by testing samples of the broth, using high-performance liquid chromatography with a UV detection system [see, for example, J.H. Kennedy in *J. Chromatographic Science*, 16, 492-495 (1978)].

Following its production under submerged aerobic fermentation conditions, tylactone can be recovered from the fermentation medium by methods used in the fermentation art. Because of the limited solubility of tylactone in water, it may not be altogether soluble in the medium in which it is produced. Recovery of tylactone, therefore, can be accomplished by

1) extraction of the fermentation broth or 2) filtration of the fermentation broth and extraction of both the filtered broth and the mycelial cake. A variety of techniques may be used in the extraction of processes. A preferred technique for purification of the filtered broth involves extracting the broth (generally without pH adjustment) with a suitable solvent such as amyl acetate or petroleum ether, con-

concentrating the organic phase under vacuum to give  
 crystals or an oil. If an oil is obtained, it may be  
 purified by adsorption chromatography.  
 The compounds of structures 1 and 2 are  
 useful intermediates from which 16-membered macrolide  
 antibiotics can be prepared. For example, ty lactone  
 (1) can be bioconverted to tylosin by adding it to a  
 growing culture of a bioconverting microorganism. The  
 bioconverting microorganism can be a Streptomyces  
fradiae strain which either produces tylosin itself or  
 is capable of producing tylosin except that it is  
 blocked in ty lactone formation.  
 A strain which is capable of producing tylosin  
 except that it is blocked in ty lactone formation can be  
 obtained by treating a tylosin-producing strain with a  
 mutagen and screening survivors for those which are  
 unable to produce tylosin. Those survivors which are  
 unable to produce tylosin are further screened to  
 determine which strains are also unable to produce  
 ty lactone. These strains are identified by adding  
 ty lactone to small shake-flask cultures of the selected  
 survivors to determine if they produce tylosin.  
Streptomyces fradiae strains NRRL 2702 and  
 NRRL 2703 are examples of Streptomyces strains which  
 are capable of producing tylosin. A typical mutagen  
 which may be used to obtain the selected strains is  
 N-methyl-N'-nitro-nitrosoguanidine.  
 The compound of structure 1 is especially  
 useful in the preparation of labeled compounds for  
 metabolic studies. By labeling either the ty lactone  
 portion or the added sugar moieties, the metabolic  
 pathway of tylosin can be ascertained.

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In order to illustrate more fully the operation of this invention, the following examples are provided:

Example 1

A. Shake-flask Fermentation of Ty lactone

5 A lyophilized pellet of Streptomyces fradiae NRRL 12188 was dispersed in 1-2 ml of sterilized water. A portion of this solution (0.5 ml) was used to inoculate a vegetative medium (150 ml) having the following composition:

<u>Ingredient</u>		Amount (%)
Corn steep liquor	1.0	
Yeast extract	0.5	
Soybean grits	0.5	
CaCO <sub>3</sub>	0.3	
Soybean oil (crude)	0.45	
Deionized water	97.25	

20 Alternatively, a vegetative culture of S. fradiae NRRL 12188 preserved, in 1-ml volumes, in liquid nitrogen was rapidly thawed and used to inoculate the vegetative medium. The inoculated vegetative medium was incubated in a 500-ml Erlenmeyer flask at 29°C. for about 48 hours on a closed-box shaker at about 300 rpm.

25 This incubated vegetative medium (0.5 ml) was used to inoculate 7 ml of a production medium having the following composition:

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# Ingredient

Beet molasses	2.0
Corn meal	1.5
Fish meal	0.9
Corn gluten	0.9
NaCl	0.1
(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	0.04
CaCO <sub>3</sub>	0.2
Soybean oil (crude)	3.0
Deionized water	91.36

Amount (%)

The inoculated fermentation medium was incubated in a 50-ml bottle at 29°C. for about 6 days on a closed-box shaker at 300 rpm.

## B. Tank Fermentation of Tylosone

In order to provide a larger volume of inoculum, 60 ml of incubated vegetative medium, prepared in a manner similar to that described in section A, was used to inoculate 38 l of a second-stage vegetative growth medium having the following composition:

# Ingredient

Corn steep liquor	1.0
Soybean meal	0.5
Yeast extract	0.5
CaCO <sub>3</sub>	0.3
Soybean oil (crude)	0.5
Lecithin (crude)	0.015
Water	97.185

The pH was adjusted to 8.5 with 50% NaOH solution.

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This second-stage vegetative medium was incubated in a 68-liter tank for about 47 hours at 29°C.

Incubated second-stage medium (4 L) thus prepared was used to inoculate 40 liters of sterile production medium having the following composition:

Ingredient  
Amount (%)

0.92	Fish meal
1.57	Corn meal
0.92	Corn gluten
0.21	CaCO <sub>3</sub>
0.10	NaCl
0.04	(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>
2.10	Beet molasses
3.15	Soybean oil (crude)
0.09	Lecithin
90.90	Water

The pH was adjusted to 7.2 with 50% NaOH solution.

The inoculated production medium was allowed

to ferment in a 68-liter tank for about 5 days at a temperature of 28°C. The fermentation medium was aerated with sterile air to keep the dissolved oxygen level between about 30% and 50% and was stirred with conventional agitators at about 300 rpm.

described in Example 1, was filtered using a filter aid (3% Hyflo Supercel, a diatomaceous earth, Johns Manville Corp.). The pH of the filtrate was adjusted to about 9 by the addition of 2% sodium hydroxide. The filtrate was extracted with amyl acetate (400 L). The amyl acetate extract (which has a high optical density reading at 282 nm but no antimicrobial activity) was concentrated under vacuum to give an oil. The oil was dissolved in benzene (5 L). The benzene solution was chromatographed over a 5.25 x 36 in. silica-gel (Grace, grade 62, Davison Chemical Co.) column, packed with benzene. Elution is monitored by silica-gel thin-layer chromatography, using a benzene:ethyl acetate (3:2) solvent system and conc. sulfuric acid spray for detection. The column was first eluted with benzene to remove lipid substances, then with benzene:ethyl acetate (9:1) to separate and isolate tylactone. Fractions containing tylactone were combined and evaporated under vacuum. Tylactone was crystallized from benzene-hexane or hot hexane to give about 2 g, m.p. 162-163°C.

The infrared absorption spectrum of tylactone in chloroform is presented in the accompanying drawing. Tylactone is a white solid which crystallizes from hexane or ethyl acetate-hexane and which melts at about 162-163°C. It has the following approximate percentage elemental composition: carbon, 70%; hydro-

#### Isolation of Tylactone

#### Example 2

30 by silica-gel thin-layer chromatography. Sulfuric acid spray, either concentrated or dilute (50%), may be used

Tylactone can be distinguished from tylosin

sulfide.

diethyl ether, petroleum ether, benzene and dimethyl

25 methanol, ethanol, dimethylformamide, chloroform,

but is soluble in organic solvents such as acetone,

Tylactone is nearly insoluble in water,

ble groups.

aqueous dimethylformamide indicates it has no titra-

20 Electrochromic titration of tylactone in 66%

$[\alpha]_D^{25} -55.23^\circ$  (c 1, CH<sub>3</sub>OH).

Tylactone has the following specific rotation:

maximum at about 282 nm ( $E_{1\%}^{1\text{cm}} = 560$ ).

15 tylactone in neutral ethanol exhibits an absorption

The ultraviolet absorption (UV) spectrum of

840 (medium), 820 (very small) and 661 (small).

923 (medium), 911 (shoulder), 859 (small), 868 (medium),

small), 1025 (medium), 984 (very strong), 958 (strong),

(strong), 1103 (medium), 1078 (medium), 1049 (very

10 (strong), 1284 (medium), 1181 (very strong), 1143

1441 (shoulder), 1404 (strong), 1379 (small), 1316

strong), 1626 (small), 1592 (very strong), 1458 (strong),

(weak), 2353 (weak), 1709 (very strong), 1678 (very

5 frequencies (cm<sup>-1</sup>): 3534 (medium), 2924 (strong), 2398

observable absorption maxima occur at the following

in chloroform is shown in the accompanying drawing.

The infrared absorption spectrum of tylactone

of C<sub>23</sub>H<sub>38</sub>O<sub>5</sub> and a molecular weight of about 394.

gen, 9.7%; oxygen, 20.3%. It has an empirical formula

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for detection. With this detection system tyllactone appears initially as a yellow-to-brown spot. If silica-gel plates with a fluorescent background are used in the chromatography, UV detection is convenient. The approximate R<sub>f</sub> values of tyllactone are summarized in Table 1.

Table 1

Thin-Layer Chromatography of Tyllactone<sup>a</sup>

Compound	R <sub>f</sub> Value	
	$\bar{A}^b$	$\bar{B}$
Tyllactone	0.50	0.62
Tylosin	0.0	0.0

<sup>a</sup>Medium: Silica gel

<sup>b</sup>Solvent: A = benzene: ethyl acetate (4:1)  
B = benzene: ethyl acetate (3:2)

Example 3

3,5-Di-O-Acetyllactone

Tyllactone (200 mg), prepared as described in Example 2, was dissolved in pyridine (4 ml). Acetic anhydride (4 ml) was added. The resulting mixture was allowed to stand at room temperature for 16 hours and then concentrated to dryness under vacuum. Methanol (5 ml) was added to the residue; the solution heated at 60° for 1/2 hour and then concentrated under vacuum to give 3,5-di-O-acetyllactone. This compound has an R<sub>f</sub> value of about 0.59 on silica-gel thin-layer chromatography in a benzene:ethyl acetate (4:1) solvent system. The R<sub>f</sub> of tyllactone in this system is about 0.3.

3,5-Di-O-propionyltylactone, prepared according to the procedure of Example 3, but using propionic anhydride.

3,5-Di-O-isovaleryltylactone, prepared according to the procedure of Example 3, but using isovaleric anhydride.

3,5-Di-O-benzoyltylactone, prepared according to the procedure of Example 3, but using benzoic anhydride.

3,5-Di-O-( $\bar{n}$ -butyryl)tylactone, prepared according to the procedure of Example 3, but using  $\bar{n}$ -butyric anhydride.

#### Example 8

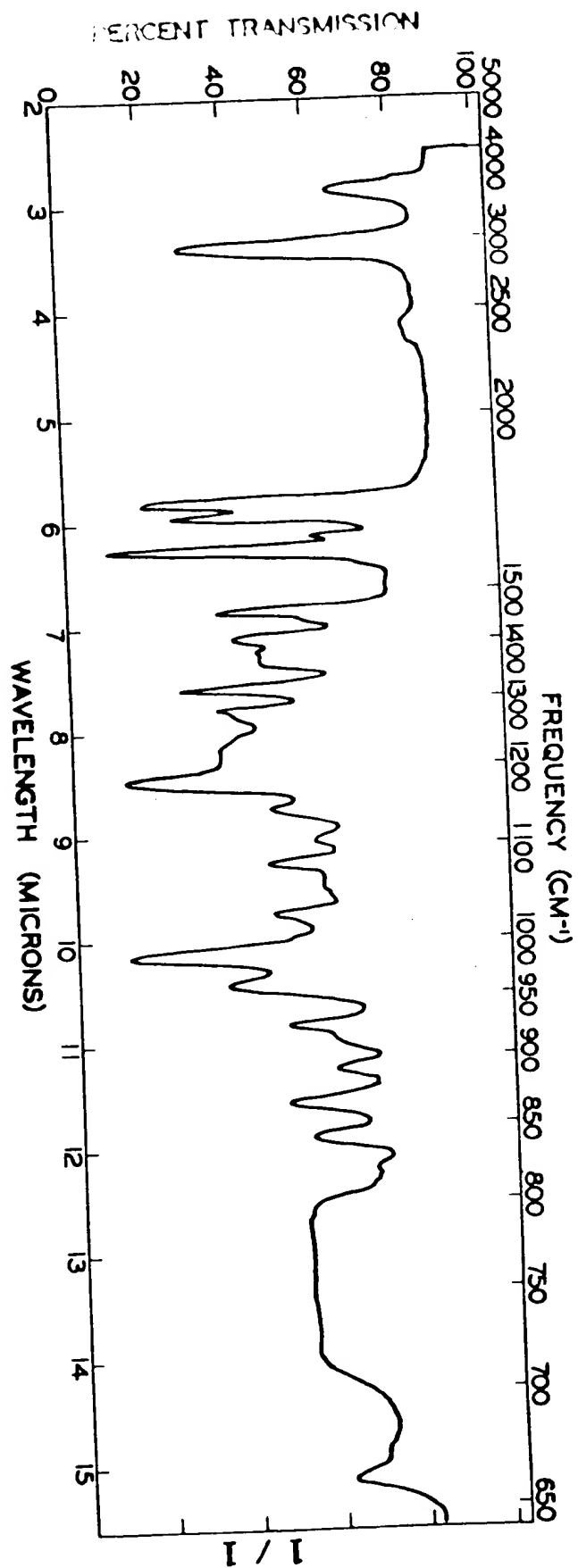
#### Preparation of Tylosin from Tylactone

A *Streptomyces fradiae* strain which formerly produced tylosin but which was blocked in macrolide ring closure was fermented according to the procedure described in Example 1, Section A, except that a temperature of 28°C was used. Tylactone was added to the fermentation 48 hours after inoculation. The fermentation was then continued until a substantial amount of tylosin was produced, i.e. about three additional days.

The presence of tylosin is determined by testing samples of the broth against organisms known to be sensitive to tylosin. One useful assay organism is *Staphylococcus aureus* ATCC 9144. Bioassay is conventionally performed by an automated turbidometric method, by thin-layer chromatography or by high-performance liquid chromatography with UV detection.

1. A process for preparing ty lactone, or an ester derivative thereof, which comprises cultivating Streptomyces fradiae NRRL 12188, or a ty lactone-producing mutant or recombinant thereof, in a culture medium containing assimilable sources of carbon, nitrogen, and inorganic salts under submerged aerobic fermentation conditions to produce ty lactone, followed, optionally, by esterification.
2. A process according to claim 1 which comprises cultivating Streptomyces fradiae NRRL 12188.
3. Streptomyces fradiae NRRL 12188.
4. A culture medium which comprises Streptomyces fradiae NRRL 12188 and assimilable sources of carbon, nitrogen and inorganic salts.
5. Ty lactone or an ester derivative thereof whenever prepared by a process according to either of claims 1 and 2.

# CLAIMS



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DOCUMENTS CONSIDERED TO BE RELEVANT		Category		X
Chation of document with indication, where appropriate, of relevant passages Relevant to claim		CHEMICAL & PHARMACEUTICAL BULLETIN, 1,2,5 vol. 28, no. 6, June 1980, pages 1963-1965, edit. by Pharmaceutical Society of Japan SATOSHI OMURA: "Isolation and characterization of a new 16- membered lactone, protylonolide, from a mutant of tylosin-producing strain, streptomycetes fradiae KA- 4271,2"		
CLASSIFICATION OF THE APPLICATION (in Cl. 9) A 61 K 31/365 C 07 D 313/00 C 12 P 17/08/ C 12 P 17/08/ C 12 R 1/54)		TECHNICAL FIELDS SEARCHED (in Cl. 9) A 61 K 31/365 C 07 D 313/00 C 12 P 17/08		
CATEGORY OF CITED DOCUMENTS X: particularly relevant A: technological background O: non-written disclosure P: intermediate document T: theory or principle underlying the invention E: conflicting application D: document cited in the application L: citation for other reasons A: member of the same patent family. corresponding document		The present search report has been drawn up for all claims Examiner		
Date of completion of the search 22-09-1981 RAJIC		Place of search The Hague		

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Application number EP 81 30 29644

**EUROPEAN SEARCH REPORT**

**European Patent**



EP 0 043 280 B1

Note: Within nine months from the publication of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European patent convention).

Courier Press, Leamington Spa, England.

CHEMICAL & PHARMACEUTICAL BULLETIN, vol. 28, no. 6, June 1980, pages 1963-1965, edit. by Pharmaceutical Society of Japan, SATOSHI OMURA: "Isolation and characterization of a new 16-membered lactone, protionolide, from a mutant of tylosin-producing strain, streptomycetes tradiae KA-427 1,2"

References cited:

(84) Designated Contracting States: BE CH DE FR GB IT LI LU NL SE

(45) Publication of the grant of the patent: 14.03.84 Bulletin 84/11

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(30) Priority: 02.07.80 US 162977

(54) Process for preparing a macrolide.

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# EUROPEAN PATENT SPECIFICATION

Europäisches Patentamt  
European Patent Office  
Office européen des brevets

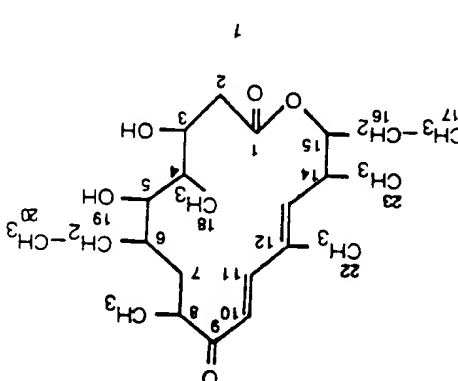
(11) Publication number:

(12)  
(19)  
(45) Date of publication of patent specification: 14.03.84  
(21) Application number: 81302964.2  
(22) Date of filing: 30.06.81  
(51) Int. Cl. 3: A 61 K 31/365, C 07 D 313/00, C 12 P 17/08 //(C12P17/08, C12R1/54)

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B1

## Process for preparing a macroliide

This invention relates to a process for the preparation of the macroliide 20-dihydro-20,23-dideoxytylone, which will be called tylactone for convenience hereinafter. Tylactone has the structure 1:



The derivatives can be prepared by esterification techniques generally known in the art, such as, for example, treatment of the compound with a stoichiometric quantity (or a slight excess) of an acylating agent, such as an acyl anhydride, in an organic solvent (for example, pyridine) at about 0°C to about room temperature for from 1 to 24 hours until esterification is substantially complete. The ester derivative can be isolated from the reaction mixture by standard procedures such as extraction, chromatography and crystallization.

Useful esters are those of organic acids including aliphatic, cycloaliphatic, aryl, aralkyl, heterocyclic, carboxylic, sulfonic and alkoxy-carbonic acids of from 1 to 18 carbon atoms, and of inorganic acids, such as sulfuric and phosphoric acids.

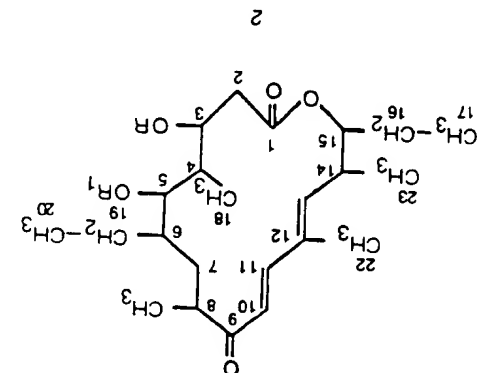
Representative suitable esters include those derived from acids such as formic, acetic, chloroacetic, propionic, butyric, isovaleric, glutaric, alkoxy-carbonic, stearic, cyclopropane-carboxylic, cyclohexanecarboxylic,  $\beta$ -cyclohexylpropionic, 1-adamantanecarboxylic, benzoic, phenylacetic, phenoxycarboxylic, mandelic and 2-thienylacetic acids, and alkyl-, aryl-, and aralkyl-sulfonic acids, the aryl- and aralkyl-acids optionally bearing substituents such as halogen, nitro and lower alkoxy on the aromatic moiety. Suitable esters also include hemi-esters derived from dicarboxylic acids such as succinic, maleic, fumaric, malonic and phthalic acids.

Tylactone can be prepared by culturing a strain of *Streptomyces fradiae* which produces this compound under submerged aerobic conditions in a suitable culture medium until a substantial amount of the desired compound is produced.

The culture medium used to grow the *Streptomyces fradiae* can be any one of a number of media. For economy in production, however, certain culture media are preferred. Thus, for example, preferred carbon sources in large-scale fermentation include carbohydrates such as dextrin, glucose, starch, and corn meal and oils such as soybean oil. Preferred nitrogen sources include corn meal, soybean meal, fish meal and amino acids. Among the nutrients inorganic salts which can be incorporated in the culture media are the customary soluble salts capable of yielding iron, potassium, sodium, magnesium, calcium, ammonium, chloride, carbonate, sulfate and nitrate ions.

Essential trace elements necessary for the growth and development of the organism should also be included in the culture medium. Such trace elements commonly occur as impurities in other constituents of the medium in amounts sufficient to meet the growth requirements.

It is useful in the preparation of related acyl derivatives which have structure 2:



The compounds of structures 1 and 2 are useful intermediates from which 16-membered macroliide antibiotics can be prepared. Although no stereochemical assignments are indicated in the structures given herein, the stereochemistry of the compounds is identical to that of tylosin.

Tylactone can be esterified at the 3- and 5-hydroxyl groups to give acyl ester derivatives by treatment with acylating agents using methods known in the art. The acyl ester derivatives of tylactone are useful as intermediates in the preparation of new macroliide antibiotics.

Typical acylating agents include anhydrides, halides (usually in combination with a base or other acid scavenger) and active esters of organic acids. Acylation can also be achieved by using a mixture of an organic acid and a dehydrating agent such as N,N'-dicyclohexylcarbodiimide. Acylations can also be carried out enzymatically using procedures such as those

chromatography with a UV detection system [see, for example, J. H. Kennedy in *J. Chromatographic Science*, 16, 492-495 (1978)].

Following its production under submerged aerobic fermentation conditions, tylosone can be recovered from the fermentation medium by methods used in the fermentation art. Because of the limited solubility of tylosone in water, it may not be altogether soluble in the medium in which it is produced. Recovery of tylosone, therefore, can be accomplished by 1) extraction of the fermentation broth or 2) filtration of the fermentation broth and the mycelial cake. A variety of techniques may be used in the extraction processes. A preferred technique for purification of the filtered broth involves extracting the broth (generally without pH adjustment) with a suitable solvent such as amyl acetate or petroleum ether, concentrating the organic phase under vacuum to give crystals or an oil. If an oil is obtained, it may be purified by adsorption chromatography.

The compounds of structures 1 and 2 are useful intermediates from which 16-membered macrolide antibiotics can be prepared. For example, tylosone (1) can be bioconverted to tylosin by adding it to a growing culture of a bioconverting microorganism. The bioconverting microorganism can be a *Streptomyces fradiae* strain which either produces tylosin itself or is capable of producing tylosin except that it is blocked in tylosone formation.

A strain which is capable of producing tylosin except that it is blocked in tylosone formation can be obtained by treating a tylosin-producing strain with a mutagen and screening survivors for those which are unable to produce tylosin. Those survivors which are unable to produce tylosin are further screened to determine which strains are also unable to produce tylosone. These strains are identified by adding tylosone to small shake-flask cultures of the selected survivors to determine if they produce tylosin. *Streptomyces fradiae* strains NRRL 2702 and NRRL 2703 are examples of *Streptomyces* strains which are capable of producing tylosin. A typical mutagen which may be used to obtain the selected strains is N-methyl-N'-nitro-nitrosoguanidine. The compound of structure 1 is especially useful in the preparation of labeled compounds for metabolic studies. By labeling either the tylosone portion or the added sugar moieties, the metabolic pathway of tylosin can be ascertained.

In order to illustrate more fully the operation of this invention, the following examples are provided:

#### Example 1

A. Shake-flask Fermentation of Tylosone  
A lyophilized pellet of *Streptomyces fradiae* NRRL 12188 was dispersed in 1-2 ml of sterilized water. A portion of this solution (0.5

ments of the organism. It may be necessary to add small amounts (i.e., 0.2 ml/L) of an antifoam agent such as polypropylene glycol (M.W. about 2000) to large-scale fermentation media if foaming becomes a problem.

For production of substantial quantities of tylosone submerged aerobic fermentation in tanks is preferred. Small quantities of tylosone may be obtained by shake-flask culture. Because of the time lag in production commonly associated with inoculation of large tanks with the spore form of the organism, it is preferable to use a vegetative inoculum. The vegetative inoculum is prepared by inoculating a small volume of culture medium with the spore form or mycelial fragments of the organism to obtain a fresh, actively growing culture of the organism. The vegetative inoculum is then transferred to a larger tank. The medium used for the vegetative inoculum can be the same as that used for larger fermentations, but other media can also be used.

The method of this invention comprises culturing a new microorganism which was obtained by chemical mutagenesis of a *Streptomyces fradiae* strain which produces tylosin. The new microorganism produces only minimal amounts of tylosin, but produces tylosone as a major component.

The new microorganism is also classified as a strain of *Streptomyces fradiae*. A culture of this microorganism has been deposited and made part of the stock culture collection of the Northern Regional Research Center, Agricultural Research, North Central Region, 1815 North University Street, Peoria, Illinois, 61604, from which it is available to the public under the accession number NRRL 12188.

As is the case with other organisms, the characteristics of *Streptomyces fradiae* NRRL 12188 are subject to variation. For example, recombinants, mutants or variants of the NRRL 12188 strain may be obtained by treatment with various known physical and chemical mutagens, such as ultraviolet light, X-rays, gamma rays, and N-methyl-N'-nitro-N-nitrosoguanidine. All natural and induced variants, mutants and recombinants of *Streptomyces fradiae* NRRL 12188 which retain the characteristic of tylosone production are a part of this invention.

*S. fradiae* NRRL 12188 can be grown at temperatures between about 10° and about 40°C. Optimum production of tylosone appears to occur at temperatures of about 28°C. As is customary in aerobic submerged culture processes, sterile air is bubbled through the culture medium. For efficient antibiotic production the percent of air saturation for tank production should be about 30% or above (at 28°C and one atmosphere of pressure). Production of tylosone can be followed during the fermentation by testing samples of the broth, using high-performance liquid

Amount (%)

Ingredient

1.0	Corn steep liquor
0.5	Soybean meal
0.5	Yeast extract
0.3	CaCO <sub>3</sub>
0.5	Soybean oil (crude)
0.015	Lecithin (crude)
97.185	Water

The pH was adjusted to 8.5 with 50% NaOH solution.

This second-stage vegetative medium was inoculated in a 68-liter tank for about 47 hours at 29°C. Inoculated second-stage medium (4 L) thus prepared was used to inoculate 40 liters of sterile production medium having the following composition:

Amount (%)

Ingredient

0.92	Fish meal
1.57	Corn meal
0.92	Corn gluten
0.21	CaCO <sub>3</sub>
0.10	NaCl
0.04	(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>
2.10	Beet molasses
3.15	Soybean oil (crude)
0.09	Lecithin
90.90	Water

The pH was adjusted to 7.2 with 50% NaOH solution.

The inoculated production medium was allowed to ferment in a 68-liter tank for about 5 days at a temperature of 28°C. The fermentation medium was aerated with sterile air to keep the dissolved oxygen level between about 30% and 50% and was stirred with conventional agitators at about 300 rpm.

(150 ml) having the following composition:

Amount (%)

Ingredient

1.0	Corn steep liquor
0.5	Yeast extract
0.5	Soybean grits
0.3	CaCO <sub>3</sub>
0.45	Soybean oil (crude)
97.25	Deionized water

Alternatively, a vegetative culture of *S. fradiae* NRRL 12188 preserved in 1-ml volumes, in liquid nitrogen was rapidly thawed and used to inoculate the vegetative medium. The inoculated vegetative medium was incubated in a 500-ml Erlenmeyer flask at 29°C. for about 48 hours on a closed-box shaker at about 300 rpm.

This incubated vegetative medium (0.5 ml) was used to inoculate 7 ml of a production medium having the following composition:

Amount (%)

Ingredient

2.0	Beet molasses
1.5	Corn meal
0.9	Fish meal
0.9	Corn gluten
0.1	NaCl
0.04	(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>
0.2	CaCO <sub>3</sub>
3.0	Soybean oil (crude)
91.36	Deionized water

The inoculated fermentation medium was incubated in a 50-ml bottle at 29°C. for about 6 days on a closed-box shaker at 300 rpm.

### B. Tank Fermentation of Tylosone

In order to provide a larger volume of inoculum, 60 ml of incubated vegetative medium, prepared in a manner similar to that described in section A, was used to inoculate 38 L of a second-stage vegetative growth medium having the following composition:



## Example 8

Preparation of Tylosin from *Tylactone*

A *Streptomyces fradiae* strain which formerly produced tylosin but which was blocked in macrolide ring closure was fermented according to the procedure described in Example 1, Section A, except that a temperature of 28°C was used. Tylactone was added to the fermentation 48 hours after inoculation. The fermentation was then continued until a substantial amount of tylosin was produced, i.e., about three additional days. The presence of tylosin is determined by testing samples of the broth against organisms known to be sensitive to tylosin. One useful assay organism is *Staphylococcus aureus* ATCC 9144. Bioassay is conveniently performed by an automated turbidometric method, by thin-layer chromatography or by high-performance liquid chromatography with UV detection.

## Claims

1. A process for preparing tylactone, or an ester derivative thereof, which comprises cultivating *Streptomyces fradiae* NRRL 12188, or a tylactone-producing mutant or recombinant thereof, in a culture medium containing assimilable sources of carbon, nitrogen, and inorganic salts under submerged aerobic fermentation conditions to produce tylactone, followed, optionally, by esterification.

2. A process according to claim 1 which comprises cultivating *Streptomyces fradiae* NRRL 12188.

## Revendications

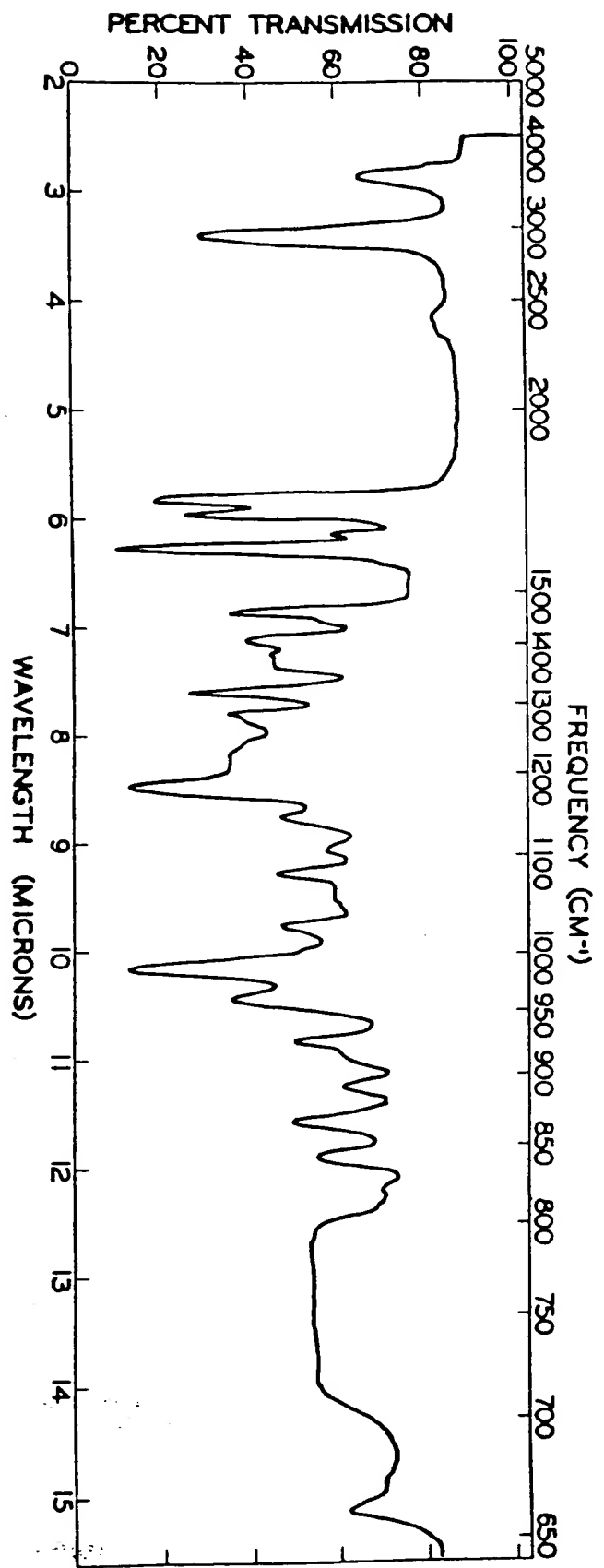
1. Procédé de préparation de tylactone ou d'un de ses dérivés esters, caractérisé en ce qu'il consiste à cultiver la souche *Streptomyces fradiae* NRRL 12188, dans un milieu de culture contenant des sources assimilables de carbone, d'azote et de sels inorganiques dans des conditions de fermentation aérobie submergée pour produire de la tylactone, cette culture étant éventuellement suivie d'une estérification.

2. Procédé suivant la revendication 1, caractérisé en ce qu'il consiste à cultiver la souche *Streptomyces fradiae* NRRL 12188.

## Patentansprüche

1. Verfahren zur Herstellung von Tylacton oder einem Esterderivat hiervon, dadurch gekennzeichnet, daß man *Streptomyces fradiae* NRRL 12188 oder eine tylactonbildende Mutante oder Rekombinante hiervon in einem Kulturmedium, das assimilierbare Quellen für Kohlenstoff, Stickstoff und anorganische Salze enthält, unter submersen aeroben Fermentationsbedingungen und Bildung von Tylacton züchtet und gegebenenfalls dann eine Veresterung vornimmt.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß man *Streptomyces fradiae* NRRL 12188 züchtet.



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